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Kaolin modulates ABA and IAA dynamics and physiology of grapevine under Mediterranean summer stress

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ABSTRACT

The foliar exogenous application of kaolin, a radiation-reflecting inert mineral, has proven to be an effective short-term climate change mitigation strategy for Mediterranean vineyards. In this work, we address the hypothesis that kaolin could improve both the hormonal dynamics and physiological responses of grapevines growing in Douro Region, northern Portugal. For this purpose, the leaf water potential, gas exchange and chlorophyll a fluorescence parameters were monitored, as well as the abscisic acid (ABA) and indole-3-acetic acid (IAA) quantification and immunolocalization were assessed. The study revealed a slight decrease in ABA and an increase in IAA in the kaolin treatment, which in turn were associated with the improvement of physiological performance. A month after spraying, kaolin improves the water potential respectively, 30% and 17% in the predawn and midday periods. Besides, plants treated with kaolin showed higher values of stomatal conductance, net CO_2 assimilation rate and intrinsic water use efficiency. Kaolin also ameliorates the effective PSII efficiency (67%), as well as the maximum quantum efficiency of photosystem II and the photosynthetic electron transport rate (> 73%). These results were consistent with the higher photochemical quenching and the lower non-photochemical quenching observed in treated leaves and with the better performance obtained by the JIP test parameters. Physiological and hormonal analysis confirmed that kaolin effectively enhance grapevine summer stress tolerance.

1. Introduction

Climatic projections, particularly for Mediterranean areas, predicts impacts on viticulture since the combined effect of high evaporative demands and drought with lengthier periods of high air temperatures during grapevine growing season might result in damaging effects, not only on productivity but also on grape and wine quality and typicity (Chaves et al., 2007; Fraga et al., 2014). At a physiological level, these stressful conditions can impose negative impacts on grapevine behavior, such as reduced leaf area and modifications in water status and photosynthesis (Moutinho-Pereira et al., 2007). The hormonal signal transduction cascades, *viz.* abscisic acid (ABA) and indole-3-acetic acid (IAA), are activated in order to maintain plasticity in growth and ability to adapt to harsh environmental conditions (Harrison, 2012; Wani et al., 2016). On one hand, ABA triggers a wide range of cellular and adaptive physiological responses (Danquah et al., 2014). Considered a dynamic hormone with multiple functions, high cellular levels of ABA enable the reduction of water loss (Finkelstein et al., 2008; Kim et al., 2010). In a climate change scenario, ABA participates as a key regulator in the activation of plant cellular adaptation strategies to abiotic stresses and has a crucial function as a growth inhibitor (Golldack et al., 2014). On the other hand, IAA is the most substantial auxin occurring in plants and it is recognized for its central roles in plant morphogenesis, including growth, development and root patterning (Zhao, 2010). Furthermore, auxins are involved in the regulation of several signaling pathways, such as cell viability, cell cycle progression and programmed

Abbreviations: *A*, Net CO_2 assimilation rate; ABA, abscisic acid; A/g_s, intrinsic water use efficiency; Ci/C_a, internal CO_2 concentration/ambient CO_2 ratio; E, transpiration; ETR, photosynthetic electron transport rate; F_v/F_m, maximum photochemical quantum efficiency of photosystem II; g_s, stomatal conductance; IAA, indole 3-acetic acid; PI_{ABS}, Performance Index of energy conservation from photons absorved by PSII antenna to the reduction of QB; q_N, non photochemical quenching; q_P, photochemical quenching; Φ_{PSII}, actual PSII efficiency; Ψ_0, Efficiency with which a PSII trapped electron is transferred from *QA* to *QB*; Ψ_{md}, midday leaf water potential; Ψ_{pd}, predawn leaf water potential

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cell death, which coordination relies on the maintenance of auxin endogenous pool at appropriate levels (Xia et al., 2005; Zhao, 2010). Environmental signals stimulate variations in IAA levels and/or its redistribution and transport in order to regulate plant growth and development. However, how auxin functions providing developmental plasticity to plants under abiotic stresses is yet to be well understood (Du et al., 2013). Despite this, the application of exogenous products can be considered to reduce the negative impact imposed by harsh climate conditions (Dinis et al., 2016a; Hasanuzzaman et al., 2013). Kaolin, a white inert mineral clay, has been shown to reduce summer stress without restricting gas exchange, whereas leaf is able to intercept photosynthetically active radiation but reflects ultraviolet and infrared radiation from the surface, reducing leaf temperature and increasing CO_2 assimilation rates (Glenn et al., 2010; Song et al., 2012). In recent works the protective effect of kaolin has been proved on photosystem II structure and function, as well as in boosting the antioxidant capacity and phenolic content in grapevines (cv. Touriga Nacional) (Conde et al., 2016; Dinis et al., 2016a; Dinis et al., 2016b). For this purpose, the main goal of this research was to study, in grapevines growing under Mediterranean field conditions, the effects of kaolin on ABA and IAA (in global quantification and at tissue leaf level), and the association with gas exchange, chlorophyll a fluorescence, water potential and total soluble protein contain.

2. Material and methods

2.1. Plant material and experimental design

The experiment initiated in 2012 and this study was carried out in 2014 at the commercial vineyard "Quinta do Vallado" in the Douro Demarcated Region (Denomination of Origin Douro/Porto) located at Peso da Régua (41°09′44.5″N 07°45′58.2″W), in northern Portugal. The climate is of the Mediterranean type with dry and hot summers, high precipitation during the winter months and very low during the summer (Fig. 1). The soil, essentially of schist origin with a loam-dominated texture, is classified as dystric-surribi aric anthrosols (FAO, 2015). The studied grapevine variety was "Touriga Nacional", a typical variety of the area, considered the finest Portuguese red grape variety, and in Douro wine region is particularly important in the production of Port wine. Grapevines were grown under dry-farming conditions and this work took place in six vineyard rows with twenty plants each. Three rows were sprayed soon after veraison (17th July 2014) with 5% kaolin (w/v) (Surround WP; Engelhard Corp., Iselin, NJ), according to previous works done by our team (Dinis et al., 2016a,b). The other three vineyard rows, were maintained as control, i.e. without kaolin application. All rows are located side-by-side (ensuring the same edaphoclimatic conditions) on a steep hill with an N-S orientation. The 7 year-old vines were trained to unilateral cordon and the spurs were pruned to two nodes each with 10–12 nodes per vine. Mature leaves (fully expanded leaves of the first third of the shoot per treatment) were sampled in two different dates after kaolin pulverization: at 23th July (one week) and 21st August (one month and half).

For biochemical analysis we used mature leaves and well exposed to sun. Samples were frozen in liquid nitrogen and stored at −80 °C, then lyophilized for 48 h and converted to a fine dried powder before analysis. For the immunohistochemistry procedure, leaves, from different individuals, were collected a month and half after application (n = 3/per treatment) and fixed (Escandón et al., 2016a).

2.2. Climatic conditions, leaf water status and leaf temperature

A meteorological station located in the field of the experiment was used to obtain weather information. Predawn (Ψ_{pd}, n = 8) and midday leaf water potential (Ψ_{md}, n = 11) were measured with a pressure chamber (PMS Instrument Company, Model 1000, Albany, USA). We took care to minimize water loss while transferring the leaf to the chamber enclosing it in a plastic bag with saturated atmosphere immediately after excision. Leaf temperature was measured with an infrared thermometer (Infratrace KM800S, England) in both periods (morning and midday) one week and one month and half after application. Measurements were performed under clear sky sunny days at the middle of the shoots (usually between 8th and 11st nodes on the shoot axes), on leaves exposed to sun and fully expanded (n = 8). The average temperature in each treatment leaves was obtained by holding the thermometer approximately 1 m above the foliar surface.

2.3. Gas exchange

Gas exchange parameters (n = 11) were determined at midday (13:30–14:30) one week and one month and half after application with a portable gas exchange system (LC*pro*+, ADC, Hoddesdon, England) before measurements of water potential. Incident photosynthetic photon flux density (PPFD) on the leaves was always greater than 1500 μmol m^{-2} s^{-1}, which is above light saturation point in this species (Flexas et al., 2002). Net CO_2 assimilation rate (A), stomatal conductance (g_s), transpiration rate (E), and internal CO_2 concentration/ambient CO_2 ratio (Ci/Ca) were calculated (von Caemmerer and Farquhar, 1981). The A/g_s ratio (intrinsic water use efficiency) was used as an estimate of the water use efficiency.

2.4. Chlorophyll a fluorescence analysis and OJIP test

Chlorophyll *a* fluorescence features were measured *in situ* (n = 11), in the same period and in the same leaves of gas exchange

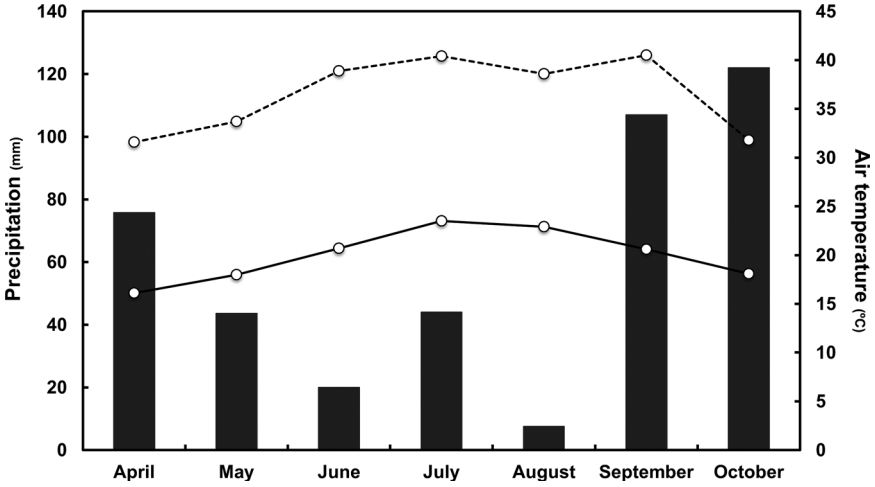

Fig. 1. Seasonal evolution of mean (full line) and maximum (dash line) air temperature and precipitation values. Data are from a nearby weather station.

measurements, by using a pulse-amplitude-modulated fluorimeter (FMS 2, Hansatech Instruments, Norfolk, England). Maximum quantum efficiency of photosystem II (PSII) was calculated as $F_v/F_m = (F_m\text{-}F0)/Fm$ by measuring the fluorescence signal on 30 min dark-adapted leaves, when all reaction centers are open using a low intensity pulsed measuring light source (F0) and during a saturating light pulse (0.7 s pulse of 15000 µmol photons $m^{-2}s^{-1}$ of white light) when all reactions centers are closed (Fm). The leaf conditions were maintained according to Dinis et al., 2014. From these measurements, several fluorescence attributes were calculated (Bilger and Schreiber, 1986; Genty et al., 1989), as the photochemical quenching ($q_P = (F'_m\text{-}F_s)/(F'_v)$, the non-photochemical quenching ($q_N = 1\text{-}(F'_v/F_v)$ and actual PSII efficiency of electron transport evaluated as Φ_{PSII} ($\Phi_{PSII} = \Delta F/F'_m = (F'_m\text{-}F_s)/F'_m$). The photosynthetic electron transport rate (Marinari et al., 2007) was estimated as ETR = $(\Delta F/F'_m)$ x PPFD x 0.5 × 0.84, where 0.5 is the factor that assumes equal distribution of energy between the two photosystems, and the leaf absorbance used was 0.84 because is the most common value for C3 plants (Bilger and Schreiber, 1986). The biophysical parameters derived from the OJIP transients were calculated according to the OJIP-test equations (Stirbet and Govindjee, 2011; Strasser and Tsimilli-Michael, 2001). The efficiency/probability, at t = 0, with which a PSII trapped electron is transferred from Q_A to Q_B was obtained as $\psi_0 = ET_0/TR_0$ and "Vitality" Index as PI_{ABS} = (RC/ABS).$(\varphi_{Po}/(1-\varphi_{Po}).(\psi_0/(1-\psi_0)$ (Performance Index of energy conservation from photons absorbed by PSII antenna, to the reduction of Q_B). The values were normalized to the control average value obtained for each chlorophyll *a* fluorescence feature. These values were set as 100%, for each feature, in the control group. The values obtained in the exposed groups were converted also as a percentage in comparison to control group. The detail formulae and terms used by the OJIP–test for analysis of the fluorescence transient O–J–I–P were given in Dinis et al., 2014.

2.5. Total soluble protein quantification

The soluble protein (SP) quantification was obtained in leaves (n = 6) also used for gas exchange and chlorophyll *a* fluorescence measurements, in order to correlate these parameters. The SP was extracted using an extraction buffer containing phosphate pH 7.5 with ethylenediaminetetraacetic acid (EDTA) and quantified according to Bradford, 1976. Summarily, after 30 min of centrifugation at 4 °C, the extract is added to the Coomassie Brilliant Blue reagent and the absorbency obtained. A calibration curve was prepared with bovine serum albumin and the results were expressed as mg g^{-1} dry weight.

2.6. Immunolocalization of ABA and IAA and stereological analysis

The immunolocalization of IAA and ABA (n = 9; three per row per treatment) were performed according Escandón et al. (2016a). Briefly, small pieces of fixed leave (0.4 cm^2) were introduced in a cryostat medium (Tissue-Tek, Killik; Sakura Finetek USA, Inc., Torrance, CA, USA) and frozen at − 23 °C. After this, sections of 50 µm were cut with a sliding cryotome CM1510S (Leica Microsystems, Wetzlar, Germany) and mounted on slides. Sections were immersed for 5 min in ascending and descending 25, 50, 75 and 100% ethanol series, washed for 30 min in Phosphate Buffer Saline (PBS) buffer containing 0.1% (v/v) Tween 20. Before incubating overnight with the ABA (Ref.: AS09 446 AGRISERA, Vännäs, Sweden) or IAA (Ref.: AS09 421 AGRISERA, Vännäs, Sweden) primary antibody, samples were pre-treated with 5% (w/v) bovine serum albumin (BSA) in PBS buffer for 30 min to reduce non-specific binding. After washing twice with 0.1% (v/v) Tween 20 in PBS buffer for 10 min, sections were incubated with Alexa 488 (Molecular Probes, Göttingen, Germany) as a secondary antibody for 1 h in darkness. Samples were washed twice for 10 min with 0.1% (v/v) Tween 20 in PBS buffer. In both immunohistochemical detection assays (ABA and IAA) negative controls were obtained by replacing the primary antibody by PBS buffer. Finally, the slides were counterstained with DAPI (4′,6- diamidino-2-phenylindole; Fluka) during 20 min and coverslipped using the mounting media Mowiol (Sigma–Aldrich Co., St. Louis, MO, USA). Fluorescence was visualized using a confocal microscope (Leica TCS-SP2-AOBS) connected to a workstation and the images were processed with Fiji Software as previously published (Schindelin et al., 2012).

Additionally, stereological analysis is an important and efficient method that can be applied in optical and confocal microscopy, being used in histology to accurately quantify, for example, the number of cells and the area and volume of biological structures or regions of an organ. The volume density (VV) is defined as the percentage of the total volume occupied by the cells marked with each selected antibody (ABA and IAA), of a well defined reference space (leaf). To determine the VV (%) of the cells presenting ABA and IAA stained within the leaf (the present reference space), a classical stereological technique was performed based on point counting (Freere and Weibel, 1968; Parkhurst, 1982) using the following formula:

$$VV\,(structure, reference) = [P(structure) \times 100] \div [k \times P(reference)]$$

where P(structure) is the number of test points within each structural component, P(reference) is the total number of test points lying over the reference space (leaf), and *k* is the ratio between the number of points on the grid used for the structure of interest and for the reference space.

Counting was done using the images captured with the confocal microscope. The stereological test grids used had two sets of points, which were previously established depending on the frequency and size of the targeted compartment, and were super-imposed on the live image of the monitor. A 1:4 grid was used, with a total of 48 coarse points targeting the reference space, and 192 points targeting all immunohistochemistry staining of ABA and IAA antibody. Point counting was made in the systematically sampled fields working with the 40 × Olympus UIS-2 objective lens (Olympus Co., Ltd., Tokyo, Japan). The first field of vision was randomly selected and the following fields were systematically sampled by stepwise movements of the stage in the x- and y-directions (220 µm × 170 µm), covering the complete section area of the leaf, in order to count all immunohistochemically stained cells.

2.7. Hormonal profiling

The extraction and analysis of the bioactive auxin, indole-3-acetic acid (IAA), and ABA (n = 8) were carried out by UPLC–MS/MS as previously described (Fleta-Soriano et al., 2015). Deuterium-labelled phytohormones were used as internal standards.

2.8. Statistical analysis

Statistical analyses were performed with Sigma-Plot 12.0 program (SPSS Inc.). Measurements were carried out in leaves collected in two different dates after pulverization: at 23th July (one week) and 21st August (one month and half). After testing for ANOVA assumptions (homogeneity of variances with the Levene's mean test, and normality with the Kolmogorov-Smirnov test), statistical differences among months and treatments were evaluated by two-way factorial ANOVA, followed by the *post hoc* Tukey's test. Significant differences were considered for $p < 0.05$. Different lower case letters represent significant differences between treatments (control *versus* kaolin) within the same month ($p < 0.05$) and *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ represent significant differences between the season (July and August) within the same treatment. Absence of superscript indicates no significant difference between treatments. As hormones analyses have only one sampling date, values were evaluated by one-way factorial ANOVA. In the specific cases of Figs. 3, 4 and 10, a one-

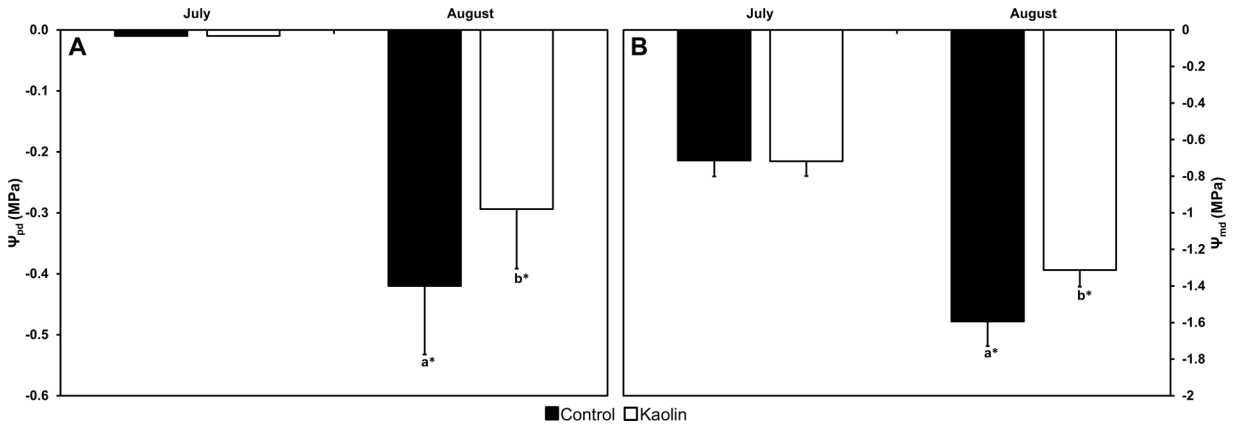

Fig 2. Predawn (Ψ_{pd}; A) (n = 8) and midday (Ψ_{md}; B) (n = 11) leaf water potential of kaolin treated plants and control ones. Statistical analysis was performed using a two-way factorial ANOVA. Different lower case letters represent significant differences between the treatment (control vs kaolin), in the same month, and * represent significant differences between months within the same treatment ($p < 0.05$). Absence of superscript indicates no significant differences.

way ANCOVA (analysis of covariance) was performed using SPSS 20.0 software. The water potential (Ψ) (Fig. 3), the carbon assimilation (*A*) (Fig. 4) and hormone quantification [ABA] (Fig. 10) were defined respectively, in each statistical analysis, as the covariate.

3. Results

3.1. Climatic conditions, leaf water status and leaf temperature

The Fig. 1 shows the air temperature and precipitation values obtained for the local under study. Maximum air temperature was always over 30 °C since April till October and mean air temperature (solid line) varied from 16.1 °C to 23.5 °C. The work period was mainly dry, except for some rainfall events at early spring and at early autumn, and light showers in mid July (one week after application). Additionally, the average of air vapor pressure deficit (VPD) was over 1.0 KPa on sampling months (one week after application − 1.22 KPa and a month and half after application − 1.18 KPa).

On the other hand, these meteorological conditions as well as the treatments affected the water potential (Ψ) (Fig. 2). One week after application, no significant differences were found in the predawn water potential (Ψ_{pd}) (Fig. 2A) neither in the midday water potential (Ψ_{md}) (Fig. 2B). However, more than a month after application (August), a significant increase in Ψ_{pd} and Ψ_{md} of kaolin sprayed plants were observed compared to July, reaching values of about 30 and 17% higher, respectively. Regarding to the leaf temperature (Suppl. 1), kaolin reduced significantly the stress heat for both months ($p < 0.05$) and day's periods ($p < 0.05$). In fact, throughout the experiment, leaves from kaolin treated plants exhibited a reduction of temperature ranging from 8.5 and 13.8 % compared to the control group.

3.2. Gas exchange parameters

The relationship between Ψ_{md} and g_s presented coefficients of determination above 0.74 one week after application and above 0.82 a month and half after application (Fig. 3). Although no significant differences was observed for Ψmd (Fig. 2B) between treatments one week after application, kaolin treated plants presents higher values of gs. More than a month after application, the Ψ_{md} is higher in kaolin treated plants also presenting higher gs (Fig. 3). Therefore, in the same sampling day, the kaolin plants showed less water stress, showing more open stomata. The ANCOVA revealed the effect of Ψ_{md}, in both July [$F(1,13) = 24.888$; $p < 0.001$] and August [$F(1,19) = 103.562$; $p < 0.001$] months, on gs. In addition, there is also effect of treatment (kaolin), in July [$F(1,13) = 30.820$; $p < 0.001$] and August [$F(1,19) = 12.243$; $p < 0.005$] on the gs after controlling the effect of Ψmd.

Meanwhile, a positive correlation between *A*/gs and *A* is observed in Fig. 4, namely a month and half after application. In general, kaolin treated plants showed higher *A*/gs and *A* values than control plants, with maximum values a month and half and one week after application, respectively, in association with the variation of water potential. The ANCOVA, in fact, revealed the non effect of *A*, in July, one week after

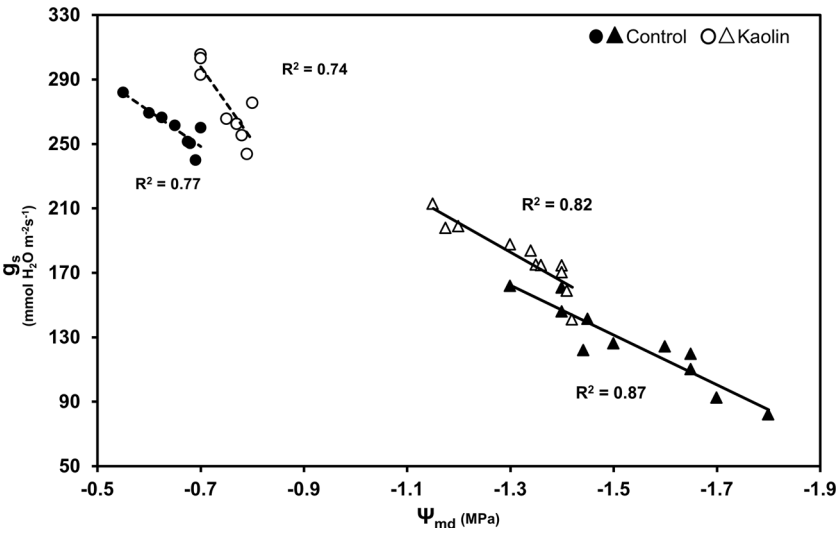

Fig. 3. Relationship between midday water potential (Ψ_{md}) and stomatal conductance (g_s) obtained in July (circles) and August (triangles) in kaolin treated plant (blank symbols) and control ones (full symbols).

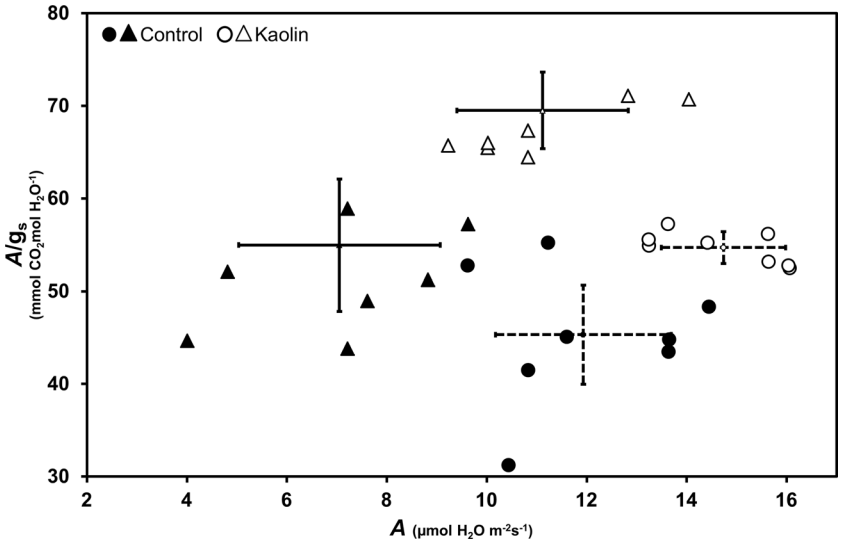

Fig 4. Relationship between net CO_2 assimilation (A) and intrinsic water use efficiency (A/g_s), obtained in July (circles) and August (triangles) in kaolin treated plant (blank symbols) and control ones (full symbols).

pulverization, [F(1,13) = 0.029; p > 0.05]; however there is effect in August [F(1,19) = 12.325; p < 0.05], on *A*. In addition, *A*/gs is affected by the treatment (kaolin), in July [F(1,13) = 6.399; p < 0.05] and in August [F(1,19) = 4.542; p < 0.05] when *A* effect is controlled.

3.3. Chlorophyll a fluorescence analysis and OJIP test

The Fig. 5 present seven parameters related to chlorophyll *a* fluorescence data and JIP parameters: effective PSII efficiency (Φ_{PSII}), electron transport rate (ETR), maximum quantum efficiency of photosystem II (F_v/F_m), photochemical quenching (q_P), nonphotochemical quenching (q_N), efficiency of energy conservation in the electron transport (Ψ_0) and performance index (PI_{ABS}). The photosynthetical light used is obtained by Φ_{PSII} and photosynthetic ETR. Concerning to Φ_{PSII}, in the midday period (Fig. 5B and D), the leaves with kaolin had higher values than leaves without the clay particles (more 17% and 67% one week and a month and half after application, respectively). This tendency was also observed in one week after application in the morning, with kaolin value 48% higher than control ones (Fig. 5A, one week after kaolin application). Concerning to ETR, kaolin plants had significant higher values than control ones except a month and half after application in the morning. The greatest difference was found in midday a month and half after application in which kaolin plants presented an increase of 73% in ETR (Fig. 5D).

Coherently, the F_v/F_m was not showing significant differences when the Φ_{PSII} and ETR values were slightly different. However, in both one week after application in the morning and one month and half after application in the midday measurements (Fig. 5A and C, respectively) kaolin treated plants presented better photosynthetical performance in both light and dark measurements.

The q_P was 19% higher a month and half after application in the afternoon in treated plants. The q_N values only presented significant differences in a month and half after application. The kaolin treated plants had 34% and 32% lower values than untreated ones, in the morning and midday periods, respectively (Fig. 5C and D). Some relative values of the JIP test parameters are shown as a multiparametric radar plot (Fig. 5; Ψ_0 and PI_{ABS}). The efficiency of energy conservation in the electron transport (Ψ_0) was higher in the kaolin treated grapevine leaves. Ψ_0 was 19% and 23% higher in the treated leaves one week after application in the morning and midday periods, respectively (Fig. 5A and B). A month and half after application, Ψ_0 kaolin leaves was 14% and 16% higher, in the morning and midday periods, respectively (Fig. 5C and D). Similar tendency appeared in the PI_{ABS}, with superior values in kaolin leaves. The major differences were found in the midday period, where kaolin leaves presented more than the double of PI_{ABS} (176% and 116% one week and one month and half after application, respectively) than control grapevine leaves (Fig. 5B and D).

3.4. Total soluble protein quantification

Total soluble proteins concentration was not affected by kaolin foliar spray one week after application in the first sampling performed (23th July), however, its effect was significant one month and half after in the second sampling (21th August), showing treated plants higher values of total soluble proteins than untreated ones (Fig. 6).

3.5. Immunolocalization and steriological analysis of ABA and IAA

The presence of immunopositive cells in grapevines leaves (Figs. 7 and 8) clearly indicate the presence of ABA and IAA; the relative volumes of these cells quantified and presented in Fig. 9A and 9B. Overall, the ABA hormone had a slightly higher distribution in mesophyll leaf tissues than vascular vessels (Fig. 7). In the control leaves, a significant higher ABA signal was observed, contrasting with kaolin ones that showed a lower signal (Figs. 7 and 9 A). Regarding to IAA signal, the relative volume of immunopositive cells was significantly higher in kaolin leaves (p < 0.01) when compared to the control ones (Figs. 8 and 9 B).

3.6. Quantification of ABA and IAA

As observed for the relative volumes one month and half after kaolin application (Fig. 9A and B), the quantification of [ABA] and [IAA] presents a similar trend (Fig. 9C and B). In fact, no significant differences were observed in the [ABA] between treated and untreated leaves (Fig. 9C), but there was a tendency (p < 0.1) for low concentration of this phytohormone in kaolin leaves. The opposite behavior was obtained for [IAA], showing the kaolin leaves more than 2.5-fold higher concentration than control ones (Fig. 9D).

There was a significant negative correlation between gs and [ABA] (Fig. 10), being for kaolin plants above 0.90. Leaves from treated plants showed the highest values of gs and the lowest values of [ABA]. The ANCOVA revealed the effect of [ABA] [F(1,13) = 57.225; p < 0.001] on the gs. In addition, there is also effect of treatment (kaolin) [F(1,13) = 39.247; p < 0.001] on the gs after controlling the effect of [ABA].

4. Discussion

According to our knowledge, this is the first study to evaluate the

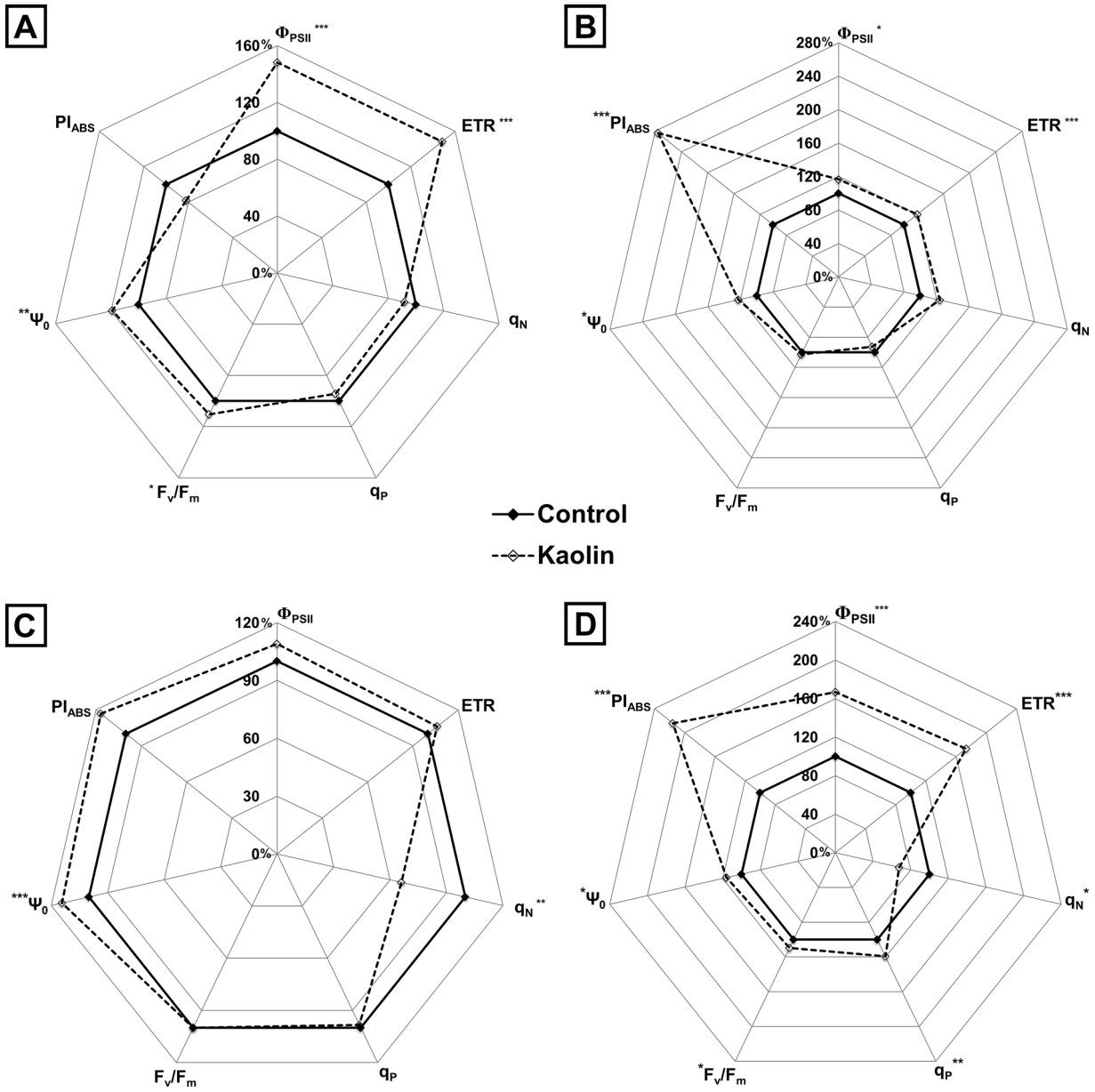

Fig. 5. Spider plot of chlorophyll a fluorescence and JIP parameters in control and kaolin treated grapevines from July morning and midday (A and B, respectively) and August morning and midday (C and D, respectively). Kaolin values (dash line) are normalized to the control ones; these values represent relative changes against the control one (full line) set as 100% for each variable. Statistical analysis was performed using one-way factorial ANOVA. Effects of kaolin were tested by Tukey's test and *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ represent significant differences between treatments (control versus kaolin). Absence of superscript indicates no significant differences.

effects on grapevine leaves of kaolin on two hormones balance, and to correlate their regulation with physiological parameters determined on grapevines growing under field conditions. This study strengthsthe starting hypothesis that kaolin constitute a short-term canopy management practice that could be applied in Mediterranean vineyards, namely for improve the physiological performance and modulates the hormonal response plants and consequently the yield quality (Dinis et al., 2016b).

Although all plants are subjected to the same soil water availability throughout the experiment (Bernardo et al., 2017), the present study showed that kaolin foliar spray improved the grapevine water relations, with significant effects on leaf gas exchange, mainly in the midday period. In fact, the short-term response to summer stress, typical in the Douro region, is the stomatal closure (Moutinho-Pereira et al., 2004), which is regulated by a complex network of signaling pathways, such as hormones (Wani et al., 2016; Zhang et al., 2006). The rapid response of hormones is essential for plant physiology and biochemistry acclimation in their environment being a critical requirement for their survival as sessile organisms (Jesus et al., 2015). As a short-term response to drought stress conditions, the hormone abscisic acid (ABA) was firstly activated (Wani et al., 2016; Zhang et al., 2006), which mediates in turn others physiological changes (Granda et al., 2011). Our findings strengthened these previous works by the increase of ABA observed in control plants, contrasting with the opposite trend in kaolin treated plants. This hormone regulates the stomatal opening/closure and the leaf growth, being synthesized in the mature chloroplasts of leaves (Pei et al., 2000) and/or in drying root, transported posteriorly via xylem also to the leaves (Correia et al., 2014; Zhang et al., 2006). These two independent paths may explain the uniform distribution of ABA in both vascular vessels and mesophyll leaf tissues, observed in this study (Fig. 7). In grapevines a direct role of ABA in stomatal conductance (g_s) upon water stress has been previously reported (Ferrandino and Lovisolo, 2014; Liu et al., 1978; Loveys, 1984). A recent vine study showed that ABA accumulation was correlated to the

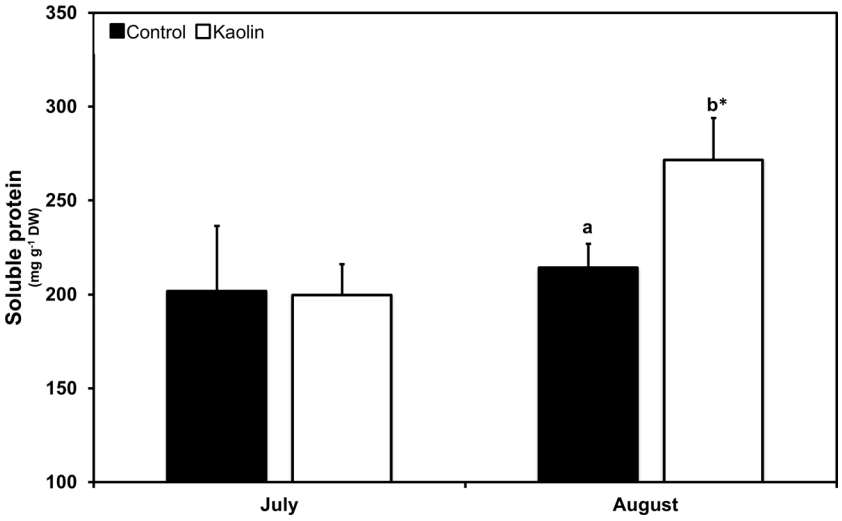

Fig 6. Leaf concentration of soluble proteins in July and August in control and kaolin treated grapevines. Values are presented as mean, n = 6/per treatment. Statistical analysis was performed using a two-way factorial ANOVA. Different lower case letters represent significant differences between the treatment (control vs kaolin), in the same month, and * represent significant differences between months within the same treatment ($p < 0.05$). Absence of superscript indicates no significant differences.

increase in leaf of osmotic potential and the decrease of gs (Carvalho et al., 2016), thus suggesting a potential signal induced by ABA synthesis and mediated by water potential (Tombesi et al., 2015). Taken together, all these findings are in agreement with our results, since the increase of ABA in the control group induced significant reduction of g_s, whereas kaolin shows an opposite trend. In fact, our data showed a strong negative correlation between gs and [ABA], being the lowest values of [ABA] observed in leaves from kaolin treated plants. This best-known phytohormone apparently interacts with other plant hormones and signaling molecules to stimulate morpho-physiological responses, as growth reduction or stomatal closure (Harrison, 2012; Wani et al., 2016). Additionally, our findings also show that the ratio between the carbon assimilation (*A*) and g_s was higher in the kaolin treated plants, indicating that g_s was more affected than *A* as reported previously by Medrano et al. (2003). Furthermore, there is a possible crosstalk modulated by ABA and auxin (IAA) levels that could affect the response of plants to drought (Sharma et al., 2015). IAA is one of the essential hormones that play important roles in regulating cell growth and development. Its levels are modulated by developmental cues, as well as by environmental signals (Sharma et al., 2015). In drought periods, the synthesis of ABA is mandatory and there are a limitation of IAA accumulation in the guard cells, which provokes the stomatal

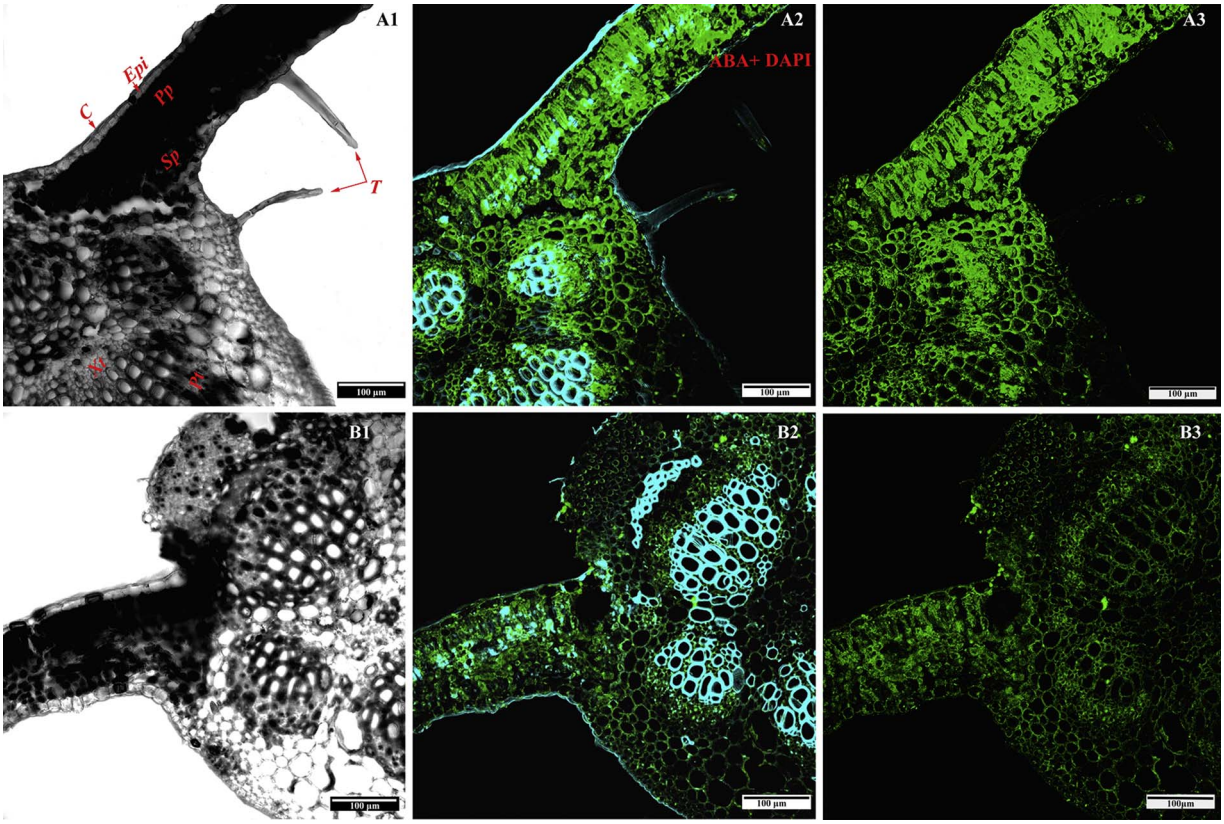

Fig. 7. Immunodetection of abscisic acid (ABA) in section of *Vitis vinifera* leaves using confocal microscope. Differential interference contrast (A1 and B1), DAPI nucleic acid stain (blue signals) (A2 and B2) and ABA (green signals) (A2, A3, B2 and B3). A1 to A3 are from leaves untreated (control); B1 to B3 are from kaolin treated leaves. Abbreviations: Epi = epidermis; C = cuticle; Pp = parenchyma palisade; Sp = spongy parenchyme; Xt = xylem tissue; Pt = phloem tissue and T = trichomes. Three biological replicates of each treatment and a negative control were performed.

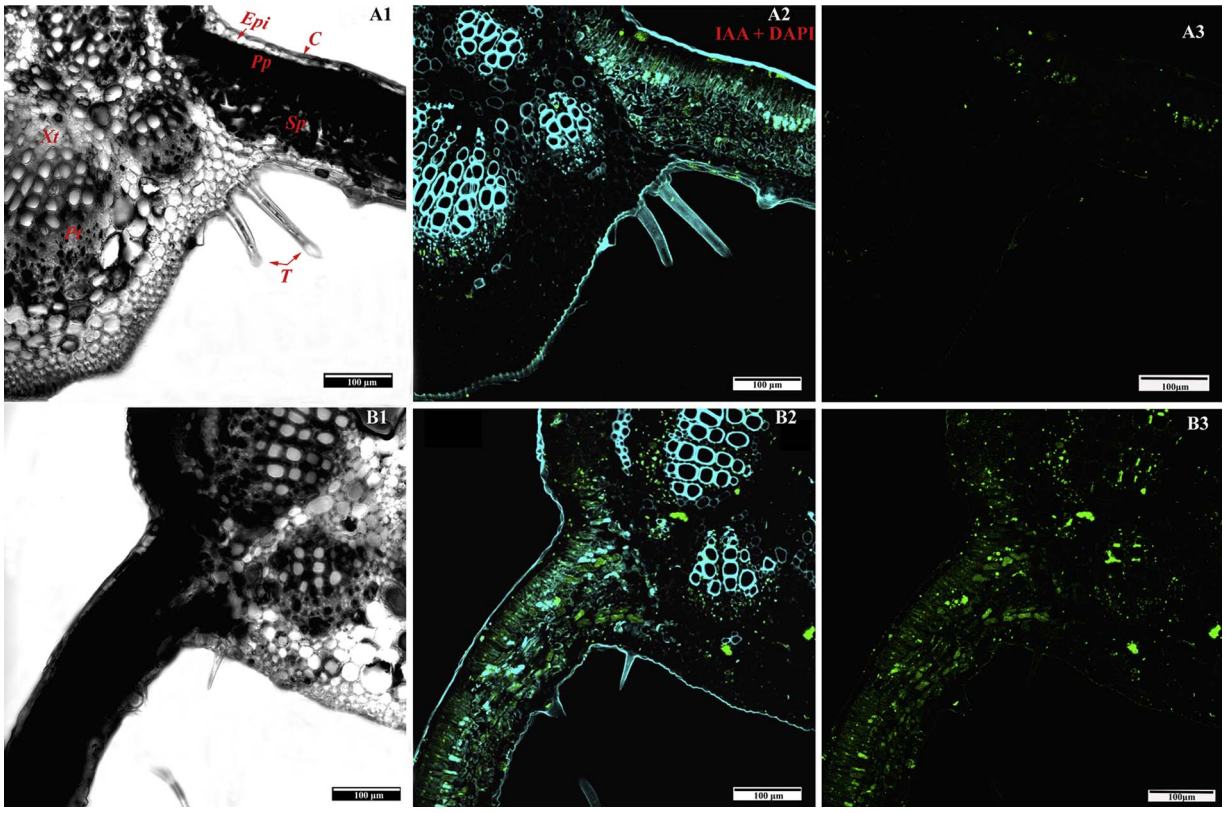

Fig 8. Immunodetection of indole-3-acetic acid (IAA) in section of *Vitis vinifera* leaves using confocal microscope. Differential interference contrast (A1 and B1), DAPI nucleic acid stain (blue signals) (A2 and B2) and IAA (green signals) (A2, A3, B2 and B3). A1 to A3 are from leaves untreated (control); B1 to B3 are from kaolin treated leaves. Abbreviations: Epi = epidermis; C = cuticle; Vv = Pp = parenchyme palissadique; Sp = spongy parenchyme; Xt = xylem tissue; Pt = phloem tissue and T = trichomes. Three biological replicates of each treatment and a negative control were performed.

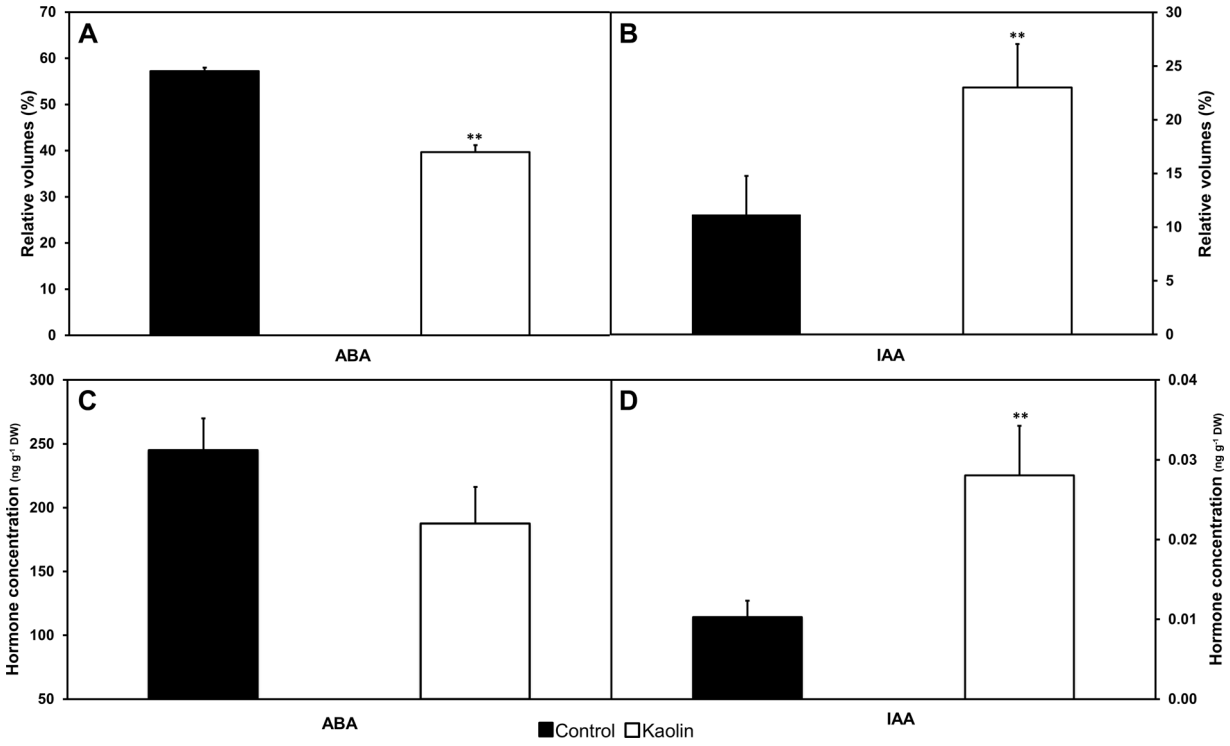

Fig. 9. Indole-3-acetic acid (IAA) and abscisic acid (ABA) concentration in kaolin treated leaves and control ones in 21 August collected in the afternoon period. Values are means ± standard deviations. Significance: ** $p < 0.01$ and absence of superscript indicates no significant differences.

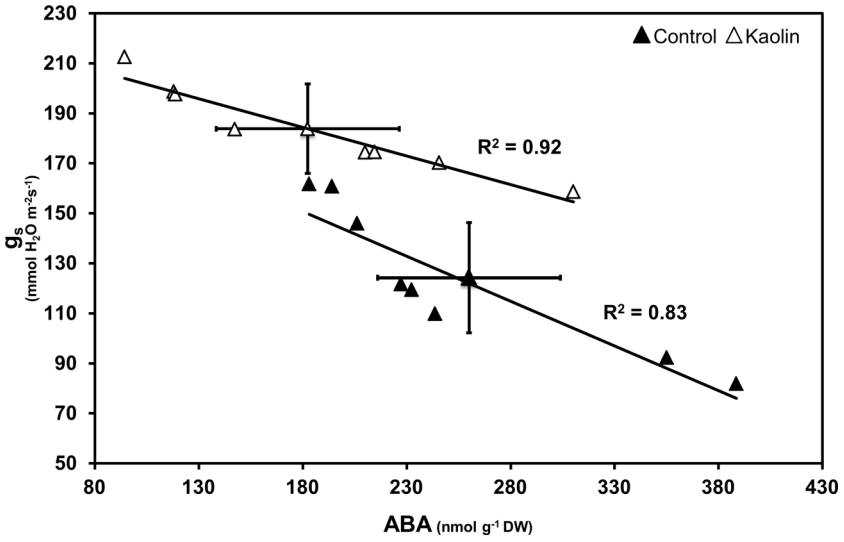

Fig 10. Relationship between abscisic acid [ABA] and stomatal conductance (g_s) obtained from the same leaves, in August during afternoon period in both kaolin treated leaves (blank symbols) and control ones (full symbols).

closure and decrease in gs. However, in better conditions (lower temperature and better water status) this tendency inverts; ABA decrease and IAA increases leading to a better plant development. The immunolocalization data indicate that IAA present an antagonist behavior compared with ABA as a response to kaolin pulverization. In fact, there was a significant increment and distribution of IAA content in treated plants, which may indicate better plant's physiological acclimation (Escandón et al., 2016b). Thus, our study corroborates the findings of previous works which reports the limit of IAA accumulation in the guard cells during drought periods (Dodd, 2005; Eckert and Kaldenhoff, 2000). This phytohormone antagonizes ABA-induced stomatal closure and when both IAA and ABA were present, stomatal aperture was dependent on the relative concentration of each one (Dunleavy and Ladley, 1995; Pospisilova, 2003; Říčánek and Vicherková, 1992). So, the overproduction of IAA, as occur in kaolin leaves, suggest an improvement of acclimation to environmental stresses in which the vines grew (Ke et al., 2015; Wani et al., 2016). Under these conditions, others metabolic pathways and processes may be stimulated. According to the differentiation/growth stability concept, phenylalanine is the same precursor of phenylpropanoid and/or protein synthesis (Koricheva et al., 1998). The precedence is the defense and the carbon converges to phenylpropanoid synthesis, as observed in our previous through the highest phenol concentrations in vines exposed to summer stress (Dinis et al., 2016b). Conversely, under adequate water availability and light conditions, the growth and development are prioritized and phenylalanine is used for protein synthesis (Koricheva et al., 1998), which explains the higher protein content in kaolin treated leaves observed in this work. Soluble proteins changes are important indicators to understand the impact of stress on cell proteolysis and protein synthesis (Santos and Caldeira, 1999). The lower values of soluble proteins in control plants could be a consequence of the hydrolysis of some proteins to response the increased request of amino acids induced by higher leaf temperature under water stress (Yadav et al., 1999).

From July to August, the kaolin treated plants present a clear increase of PSII photochemical efficiency, expressed by higher values of F_v/F_m and Φ_{PSII}. These results, together with the lower q_N and higher q_P and ETR values, suggested an improvement of regulation of electron transport, being enough to match the demand for electrons through NADP+ consumption (DaMatta et al., 2002). The higher q_N value of control plants, observed in this work, must be correlated to the induction of a higher dissipation of the excess energy by heat to avoid photosynthetic damages due to the oxidative stress (Baker, 2008). On the other hand, the lower F_v/F_m, Φ_{PSII} and ETR values in control leaves indicate that the total electron flow through PSII was more inhibited, which are in accordance to leaf gas exchange results. The reduction of PSII efficiency was also observed in response to water stress (Epron et al., 1992) and high leaf temperature, which kaolin seems to mitigate according to the results described above. Ψ_0 is a parameter related to photosynthetic capacity and is proportional to the amount of PSII centers capable to move electrons to QA (Antal et al., 2009). The kaolin application raises the Ψ_0 value, which may enhance the electron transport between QA and QB. Additionally, the similar trend in kaolin treated plants for F_v/F_m, Ψ_0 and ETR indicate a strength association between the amount of photochemically competent P_{SII} (F_v/F_m) and capability to transfer electrons to QA (Ψ_0). Furthermore, between one week and a month and half after application, the energy fluxes (Ψ_0), F_v/F_m and ΦPSII decrease in control plants, may be due to the increase of heat stress effect. The JIP-test analysis provides various fluorescence parameters (Strasser et al., 2004) and offers more knowledge about photosynthetic procedures, and subsequently about "vitality" of stressed plants. One of the most sensitive JIP expressions is the PI_{ABS}. This parameter involves fluorescence transient changes linked with alterations in antenna conformation and energy oscillations. It provides a way of establishing vitality categories and distinguishing plants based on their photosynthetic behavior (Hermans et al., 2003). The PI_{ABS} was positively affected by kaolin application, mainly in midday period. The reduction of plant "vitality" (PI_{ABS}) observed in untreated plants was influenced by the relative yields (such Ψo) and probably not by the inactivation of RCs.

This study shows that kaolin has a significant influence on the physiological behavior of grapevines (Fig. 11). The clear impact of this treatment is visible on the increased leaf reflectance, lowered PAR absorbed and leaf temperature (Dinis et al., 2017) that provokes a reduction in the VPD (Jifon and Syvertsen, 2003), which is probably related with the lowered transpiration (E) (Suppl. 2) and higher water potential. These finding together leads to an increase of *A*, g_s and the ratio A/g_s, also known as water use efficiency (WUE); which in turn influenced the relationship between two of the most important hormones in the growth and defense processes against environmental stresses typical of Mediterranean regions.

5. Conclusion

In the present work, we showed that physiological performance of the kaolin treated plants mitigated stress more than the control in the harshest season of the year. Kaolin application on the leaf provokes higher sunlight reflection, leading to lower leaf temperature and thus better leaf water status, deeply associated to the lower accumulation of ABA. Therefore, the treated plants showed higher g_s, being this response helpful against summer stress. The knowledge of these

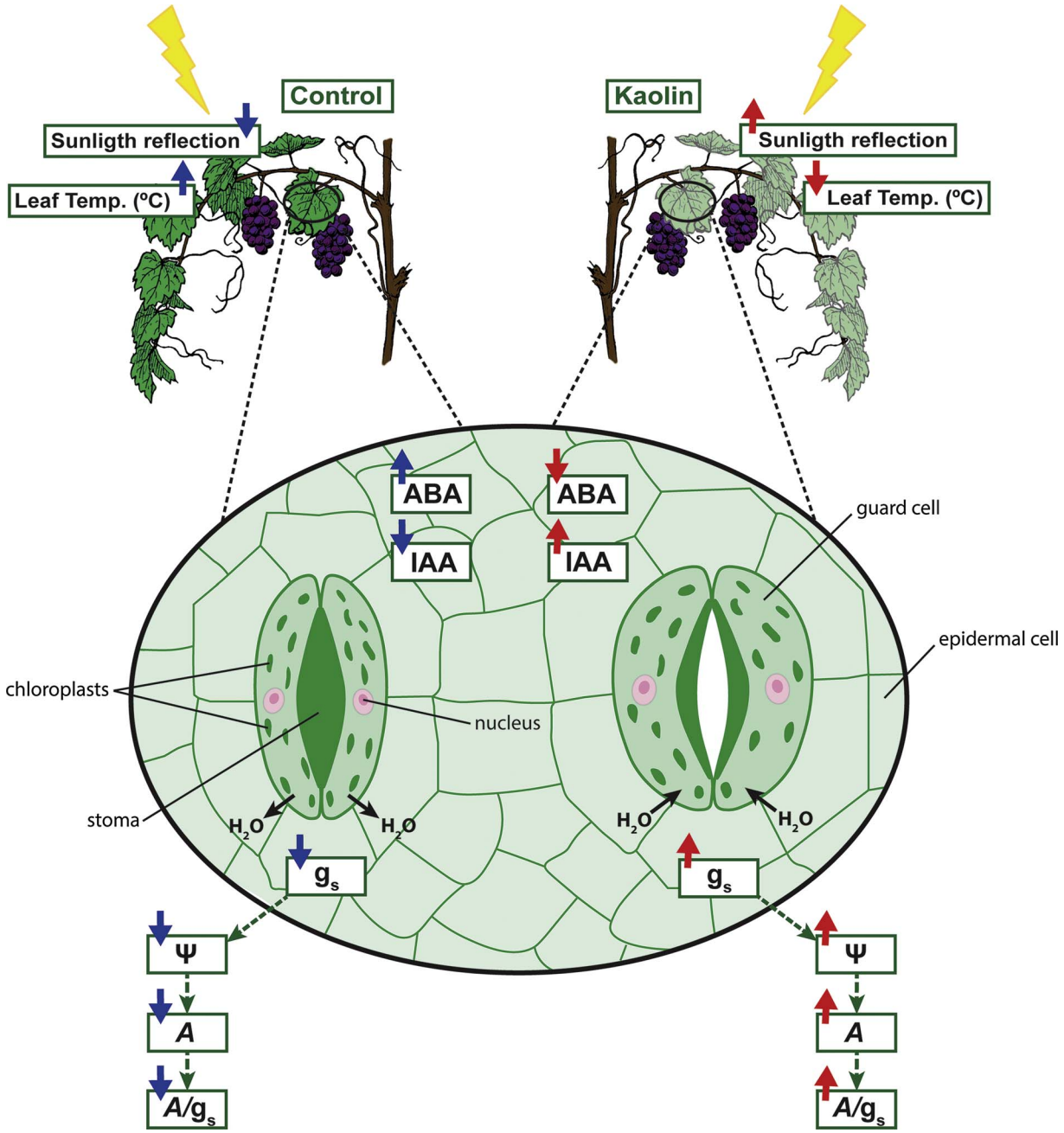

Fig. 11. Schematic representation of control (blue arrow) and kaolin (red arrow) treatment in grapevine (*Vitis vinifera*) leaf based in our physiological and ABA/IAA hormonal dynamics results. *A* – carbon assimilation; ABA – abscisic acid; g_s – stomatal conductance; IAA –indole-3-acetic acid; and Ψ – water potential.

mechanisms of mitigation will support the application of this particle film in Mediterranean viticulture environments. Regarding to the physiological, biochemical and hormonal responses, the knowledge of these strategies of adaptation may also support this agronomical technique taking in consideration future climatic conditions in the Douro region.

Author contributions

The work presented here was carried out in collaboration between all authors. Correia, C.M., and Moutinho-Pereira, J. defined the research theme. Dinis, L.-T. and Pinto, G. designed methods and experiments. The fieldwork was done by Dinis, L.-T., Bernardo, S., Correia, C.M. and Moutinho-Pereira, J. Dinis, L.-T., Bernardo, S., Meijón, M., Pintó-Marijuan, M., and Cotado, A. carried out the laboratory experiments and Dinis, L.-T., Bernardo, S., Luzio, A. analysed the data, interpreted the results and wrote the paper. All authors have contributed to, seen and approved the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jplph.2017.11.007.

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